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The Cerebroside Sulfate Activator from Pig Kidney: Purification and Molecular Structure

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The activator protein for hydrolysis of cerebroside sulfate by arylsulfatase A was purified from pig kidney in high yield. This protein, also known as sphingolipid activator protein-1 and saposin-B, was particularly rich in pig kidney. Purification was achieved by a simple procedure involving homogenation and heat treatment followed by affinity, ion exchange, and gel filtration chromatographies. The final product was better than 90% pure by gel electrophoresis and HPLC. It was possible to sequence more than 60 amino acids from the N-terminus with only a few uncertain residues. The sequence differed from that predicted for the human protein by about 10%, with most amino acid variations being conservative. There appeared to be a residual glycosyl substituent on asparagine 21, but the sugar content was low and the protein failed to bind to concanavalin A. The cerebroside sulfate activator proved to be exceptionally resistant to denaturation or protease digestion. The apparent molecular mass was ~20,000 Da on preparative gel-filtration columns, but was variable when estimated by HPLC gel filtration. Values ranging from 30,000 to over 100,000 Da were observed in neutral buffers, while values around 15,000–16,000 Da were seen in acidic buffers such as those used for assay of the biological activity. This was further decreased to a putative subunit of 7000–8000 Da under severe denaturing conditions. Pig kidney is a convenient source for the large-scale preparation of this interesting protein which has heretofore been obtained from human sources.

It was first noted by Mehl and Jatzkewitz (1) that hydrolysis of cerebroside sulfate (CS) by arylsulfatase A was dependent on a second heat-stable protein "factor." This factor which came to be referred to as cerebroside sulfate activator (CS-Act) has in recent years been shown to be identical with the GM_1 and globotriaosylceramide activators (2) and has been designated sphingolipid activator protein 1 (SAP-1) by Inui *et al.* (3). Dewji and co-workers isolated a cDNA for this protein (4) and eventually were able to show that it was a part of a larger precursor protein having three additional domains of similar structure (5). Each

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of these has proven to be activator proteins for other sphingolipid hydrolase systems (6). This multifunctional precursor protein has been called prosaposin with the individual activators designated as saposin A to D (7). Under the saposin nomenclature, the cerebroside sulfate activator is saposin B. We will retain the designation cerebroside sulfate activator (CS-Act), since it is through this activity that the protein has been purified and characterized. The structure of the human prosaposin gene was recently defined by Holtschmidt *et al.* (8). The CS-Act sequence is contained within a multiple exon segment of about 0.6 kb length.

CS-Act is a heat stable protein of relatively low molecular mass (about 20,000 Da) (9,10). It appears to function by binding, and thereby solubilizing, the respective lipid substrates making them available to water soluble hydrolytic enzymes (11). Evidence suggests that this activator in its mature form occurs primarily in lysosomes (12). While there had been some concern that such activators might be relatively nonspecific lipid-binding proteins, their biological relevance and specificity has been reaffirmed by the discovery of activator deficiency syndromes (13).

Three disease-related mutations in the human CS-Act protein have recently been reported (14–17). A possible polymorphism involving the insertion of two or three additional amino acids was suggested by the recovery of multiple cDNAs but such variations have not yet been confirmed by protein sequencing (17,18). The underlying mRNA polymorphism appears to be due to ambiguity in splice site recognition (18).

While the initial description of CS-Act by Mehl and Jatzkewitz utilized pig kidney (1), most subsequent studies have involved material isolated from human liver. Conzelmann and Sandhoff have recently reviewed preparative methods for glycolipid activator proteins including CS-Act (19). When we were in need of a reliable supply of CS-Act for fibroblast correction studies we decided to reevaluate nonhuman sources which could easily be obtained commercially. Pig kidney proved to be an exceptionally good starting material with an initial specific activity several-fold higher than that in human liver or other animal tissues investigated. The present paper describes a relatively simple purification of pig kidney CS-Act and some chemical and physical characterizations of the molecule.

METHODS

Materials. Pig kidneys and other animal tissues were obtained from a local meat company. Arylsulfatase A (ARSA) for activator analyses was a high specific activity "peak-edge" fraction obtained during the purification of pyrogen-free enzyme from human liver (20). The enzyme was diluted to 250 U/ml with 50 mM Tris–Cl, pH 7.5, containing 1 mg/ml fatty acid-free bovine serum albumin (BSA). One unit of ARSA hydrolyzes 1 μmol *p*-nitrocatechol sulfate per hour under an adaption of the assay of Baum *et al.* (21).

Cerebroside sulfate from bovine brain was purchased from Supelco (now Matreya) and mixed with [^{35}S] rat brain CS prepared in our laboratory (22). The lipids, dissolved in chloroform:methanol (2:1, containing 5% water) were mixed, dried under nitrogen, and redissolved in 25 μl of the same solvent. Water at 90–95°C was added with rapid stirring to provide a 1 mM CS suspension with $>10^3$ cpm ^{35}S/nmol. Essentially all organic solvent was volatilized during this initial

dispersal process. After stirring at room temperature for 2 h the vessel was placed in a bath sonicator at 80°C for an additional 2 to 3 h. The nearly translucent CS suspension was stable on storage and provided reasonably reproducible behavior in the various cerebroside sulfatase assays.

CS-Act assay. The basic CS-Act assay was evolved from those originally introduced by Fischer and Jatzkewitz (9). The standard 60 μl reaction took place in a 1.5-ml microcentrifuge tube and contained 2.5 units ARSA, 10 nmol [^{35}S]CS (>10^4 cpm) and 0.167 M sodium acetate–acetic acid buffer, pH 4.5. Approximately 30 mM Tris–Cl and 10 μg BSA were present from enzyme and activator additions. Samples were mixed, incubated at 37°C for 4 h, and the reaction stopped by the addition of 1 ml of chloroform:methanol (2:1) followed by 200 μl of salt–sulfate solution (4 mM Na_2SO_4 in 0.73% NaCl). The [^{35}S]sulfate released was then determined by a Folch-type extraction protocol (23). For quantitation of CS-Act activity several dilutions of activator were assayed and specific activity was based on an interpolated dilution which would yield 10% hydrolysis of the substrate (1 nmol). This was within the range of linear dependence on activator concentration. This normalization of activator dilution was critical for comparison of various fractions because of the high sensitivity of this reaction to protein inhibition. Specific activity was expressed as nmol CS hydrolyzed/mg protein/4 h under these particular conditions of assay. (This is the unit originally defined by Mehl and Jatzkewitz and differs from that employed by Mitsuyama (10) by a factor of 4.)

Comparison of sources for large-scale CS-Act preparation. Fresh porcine and bovine liver and kidney samples were minced after being freed of fat, obvious blood vessels, and connective tissue. Samples of 100 g were added to 150 ml cold water and homogenized for 2–3 min in a Waring blender. The homogenates were rapidly heated to >90°C in a boiling water bath and kept at this temperature for 5 min. Extracts were subsequently cooled on ice and centrifuged at 8000 rpm (10,000*g*) for 10 min. Supernatants were decanted and stored overnight at 4°C. Extracts were then filtered through Whatman No. 1 to remove fat which had solidified on the surface. The filtrates were concentrated to a protein concentration of ~5 mg/ml, dialyzed against 50 mM Tris–Cl, pH 7.5, and assayed. CS-Act activity could not be demonstrated in most samples before dialysis.

CS-Act preparation from pig kidney. The procedure described reflects the most recent of a number of preparations and has evolved somewhat over time. Whole pig kidneys from freshly butchered animals were freed of capsule and excess fat and ground in a commercial market meat grinder. Ground kidney was packaged in 500-g portions and frozen (−20°C). Frozen material remained fully active for more than 1 year.

Ground kidney samples were thawed and homogenized in a 4-liter Waring blender with 1.5 times their weight of water (1.5 liters/kg). Blending was for two 1-min periods at low speed, 1 min at high speed, and an additional 1 min at low speed. The homogenate was divided into ~500-ml aliquots in 1 liter Erlenmeyer flasks. These were heated with gentle stirring in an actively boiling water bath and extract temperature maintained above 90°C for 5 min. It is critical that heating occurs rapidly. Heat-treated extracts were cooled and centrifuged (500 ml Sorvall bottles with caps) for 20 min at 9000 rpm (13,600*g*). Supernatants were carefully

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decanted and stored overnight in the cold. An additional 15% activity could be recovered by washing the pelleted residues. Extracts were subsequently filtered through Whatman No. 1 on a Buchner funnel to remove solidified fat. An aliquot was dialyzed against 50 mM Tris–Cl, pH 7.5, for analyses. Protein was typically in the range of 3 to 5 mg/ml and CS-Act activity between 15 and 20 U/mg protein.

Con A/octyl Sepharose affinity chromatographies. A 2.5 × 20 cm column (100 ml bed) of Pharmacia Con A Sepharose was preconditioned by elution with 60 ml of 0.3 M α-methylmannoside, 0.3 M NaCl in buffer, washing with 0.3 M NaCl in buffer, and equilibrating with 50 mM Tris–Cl, pH 7.5. At the same time, a 2.5 × 22.5 (110 ml bed) column of octyl Sepharose 4B (Pharmacia CL-4B), which had been regenerated according to the manufacturer's recommendation, was also equilibrated with the buffer. The two columns were connected in tandem so that the effluent from Con A Sepharose flowed directly onto the octyl Sepharose. After an additional period of buffer washing, 0.5 to 1 liter of heat-treated pig kidney extract was applied at a flow rate of 1–2 ml/min. This was followed by buffer until the 280-nm absorption returned to baseline (0.5 to 1 liter). While the loading parameters were within the reasonable capacities of these affinity materials (i.e., the octyl Sepharose did not show breakthrough of CS-Act until about 40 column volumes of kidney extract had been applied) a variety of plumbing and column problems were often experienced. The extracts were usually somewhat opaque, but clarification by various filtration and/or centrifugation pretreatments did not prove to be worth the effort with these large extract volumes. Reasonably diligent column monitoring over the nearly 24-h column loading period was therefore important to avoid column plugging and other flow problems.

After washing, the columns were disconnected and eluted separately. Octyl Sepharose-bound material was eluted with octyl β-D-glucopyrannoside. Both gradient and frontal elution protocols have been employed. The gradient protocol involved a linear increase in detergent from 0–25 mg/ml in buffer over a total volume of 400 ml. CS-Act eluted early in the gradient. For frontal elution, 1 column volume of 25 mg/ml octyl glucoside in buffer was applied, with 2.5% glycerol included in the final 2 ml. This was followed by buffer until baseline 280 nm absorption was achieved. Aliquots of selected fractions were dialyzed to remove octyl glucoside and CS-Act was analyzed. CS-Act rich fractions were pooled.

The Con A Sepharose column was eluted with 1 column volume (100 ml) of 0.3 M α-methylmannoside in buffer containing 0.3 M NaCl. The final 2.0 ml also contained 2.5% glycerol. This was followed by buffer until baseline 280 nm absorption was reached. Gradient elution has been employed but did not significantly improve specific activity in the active eluate pools. Aliquots of selected column fractions were dialyzed and assayed. Active fractions were pooled and frozen. Con A-bound material from several preparations was eventually pooled, applied to an octyl-Sepharose column, and processed through the same procedure described for the unbound component.

DEAE-Sephadex chromatography. DEAE-Sephadex A-50 was preconditioned according to the manufacturer's directions and packed as a 2.5 × 21 cm column by upward flow at 1–2 ml/min. The pooled octyl-Sepharose fraction (50–100 ml) was applied at a flow rate of 1 ml/min, followed by buffer until the 280-nm

absorption of the eluate returned to baseline. Flow through the column was then reversed and CS-Act eluted with a linear gradient of 0 to 0.5 M NaCl in buffer over 4–5 column volumes (400–500 ml). (Stepwise protocols were employed in some preparations; for example, in 1–2 column volumes of 50 mM NaCl in buffer followed by 0.5 M NaCl in buffer, the CS-Act was eluting sharply at the salt transition zone.) Fractions were assayed directly and active fractions were pooled. When careful monitoring of specific activity was desired, aliquots were removed and dialyzed before analysis since salt at the elution concentration of ~0.3 M NaCl was somewhat inhibitory. Active fractions were pooled (~250 ml) and concentrated to less than 10 ml.

S-300 gel filtration. A 2.4 × 82 cm column (370 ml bed volume) of Sephacryl S-300 HF was flow-packed with buffer at 0.4 ml/min. (A 5 × 125 cm column flowing at 2 ml/min and a 25 ml sample volume was used in some preparations.) The column had been standardized by application of a mixture of 5 mg each of Blue Dextran (Pharmacia), myoglobin, and L-tyrosine in 5 ml buffer. The DEAE-Sephadex pool which had been concentrated to 6–7 ml was then applied. Fractions were directly assayed for CS-Act and those containing more than 5% of the peak activity were pooled, concentrated to between 5 and 10 mg/ml, and stored frozen. For procedures where Tris or other buffer salts would interfere, aliquots were dialyzed against water.

Acrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was carried out with a 10% acrylamide separating gel and a 5% stacking gel using the buffer system of Laemmli (24). Gels were stained using the BioRad silver staining kit according to the manufacturer's directions except that the first fixation step was done in 100 ml of 25% methanol and 8% acetic acid to which 0.6 ml of 50% aqueous glutaraldehyde had been added. For the nondenaturing "native" electrophoresis a 12% acrylamide gel was employed with a continuous Tris–borate, pH 8.3, buffer system. The buffer concentration was 90 mM in the running gel, 60 mM in the stacking/sample gel, and 90 mM in both electrode chambers. One portion of the gel was fixed and stained in a mixture of 50 ml methanol, 16 ml acetic acid, 134 ml water, 1.2 ml of 50% aqueous glutaraldehyde, and 0.3 g Coomassie blue R-250 for 2 h. The gel was then washed in 25% methanol, 8% acetic acid for several hours. Lanes from the other portion of the gel were divided into 0.5-cm strips which were eluted for 20 h with 0.45 ml of 50 mM Tris–Cl, pH 7.5. After removing the gel fragments by centrifugation the CS-Act activity was determined on 30 μl of the extracts.

Reverse phase HPLC analysis. CS-Act (100 μg) was analyzed by reverse phase HPLC using a 25 × 0.4 cm Vydac 10 μm C_{18} column at a flow rate of 1 ml/min with a programmed gradient from 0.1% TFA to 0.1% TFA in 80% acetonitrile over a period of 30 min. Fractions (0.5-ml) were collected, dried under vacuum, and reconstituted in water. Aliquots were then analyzed for protein and CS-Act activity. CS-Act peak fractions were pooled for carbohydrate analysis.

Amino acid and sequence analyses. Total amino acid and amino acid sequence analyses were carried out by the UCLA Protein Microsequencing Laboratory. Protein was estimated from the total amino acid yield corrected for the expectation that cysteine would constitute 7.4% of the total amino acids and that tryptophan

was absent. Samples were dialyzed against water, dried under vacuum, and hydrolyzed in 6 N HCl under nitrogen for 18 h at 110°C. This was followed by derivatization with phenylisothiocyanate and chromatography on a C_{18} reverse phase HPLC system. Recoveries were estimated from concurrent hydrolysis/derivatization of a standard amino acid mixture. Sequence analyses were carried out with an Applied Biosystems 475A gas-phase sequenator employing Edmund chemistries according to Hewick *et al.* (25). The phenylthiohydantoin derivatives were estimated by on line analysis.

Carbohydrate analyses. Carbohydrates were estimated by the procedure of Hardy *et al.* (26) using the Dionex pulsed amperometric detection system. Samples were dialyzed into water, dried under vacuum, dissolved in 2 M trifluroacetic acid (TFA), and hydrolyzed at 100°C for either 3 or 6 h. Hydrolysates were dried, placed in an evacuated desiccator over sodium hydroxide flakes for 18 h, redissolved in water, and the drying procedure repeated to ensure complete removal of TFA.

After redissolving the residue in water, appropriate aliquots were applied to a Dionex AS-6 column. Elution was with either 15 or 20 mM NaOH (isocratic) at a flow rate of 1 ml/min. (Glucose and mannose were not resolved at 20 mM, while glucosamine and galactose were difficult to differentiate at 15 mM.) Sorbitol was added before hydrolysis as an internal standard to correct for variation in sample recovery, injection volume, and detector sensitivity. Peak areas were compared with standard mixtures run at the start and finish of each set of samples. Neutral sugars were estimated from 3-h and amino sugars from 6-h hydrolyses if there were discrepancies.

Sialic acid was assayed on unhydrolyzed samples by the resorcinol method (27).

Dialysis and ultrafiltrations. All dialyses were carried out with low molecular weight cutoff Spectra/Por 3 membranes. These were treated to remove glycerol and sulfides by heating to 80°C for 1 min in 0.1% sodium sulfide, rinsing with water at 60°C for 2 min, and then adding 4 ml of 0.3% H_2SO_4 to each 100 ml of wash. Membranes were thoroughly rinsed with water and stored in 10% ethanol at 4°C. The use of standard dialysis membranes can result in considerable loss of CS-Act at the later stages of the purification.

CS-Act was retained on PM-10 (Amicon) ultrafiltration membranes early in the preparation, but loss through these membranes was frequently encountered with more purified material. Losses were particularly serious when filtrations were carried out under mildly acidic or basic conditions. With pH 4.5 acetate buffer, no activator activity was retained by PM-10 membranes. Similar results were obtained at pH 9.0 and above. Complete retention under all conditions evaluated was achieved with YM-2 membranes which have a 3000-Da cutoff. YM-2 membranes were eventually adopted for all CS-Act concentration protocols. Unappreciated loss of CS-Act on dialysis and concentration seems to have been largely responsible for the poor yields often encountered in earlier preparative studies.

Lyophilization. Pig kidney activator was stable to lyophilization if drying was complete. While we normally maintained laboratory stocks of CS-Act frozen in 50 mM Tris–Cl, pH 7.5, the protein was usually dialyzed and lyophilized for shipping or chemical analyses.

TABLE 1
CS-Act in Crude Heat-Treated Tissue Extracts

	Bovine		Porcine	
	Kidney	Liver	Kidney	Liver
CS-Act sp act unit/mg protein	1.1	2.1	7.6	0.44
Unit/g tissue	3.1	5.1	23.8	1.6

Note. CS-Act is inhibited under these conditions and actual values may be two- to fivefold higher.

Protease susceptibility. To 0.55 mg of CS-Act in 100 μl of 50 mM Tris–Cl was added 50 μl of 11 mg/ml Pronase (Calbiochem) in the same buffer, yielding a 1:1 mixture of activator and protease. The mixture was centrifuged for 2 min (Eppendorf microfuge) and the supernatant incubated at 55°C over a 24-h period. Aliquots were removed for CS-Act and protein analyses at various times.

Molecular weight by HPLC gel filtration. A TSK G-2000 SW column (7.5 × 300 mm) (BioRad) was equilibrated with appropriate buffer and 50 μl of a 1 mg/ml solution of CS-Act in buffer was injected. The column was eluted with buffer at 0.5 ml per min and absorbance at 280 nm was monitored. When appropriate, fractions were collected at 1-min intervals and assayed for protein and CS-Act. Standards included ovalbumin, myoglobin, carbonic anhydrase, ribonuclease A, cytochrome C, insulin, glucagon, bacitracin, and tyrosine.

RESULTS

A comparison of CS-Act activities in heat-treated extracts of commercially obtained tissues is presented in Table 1. These values can be compared with specific activities between 0.5 and 1.5 U/mg in comparable human liver extracts (Louis *et al.*, manuscript in preparation). Pig kidney contains 5- to 10-fold higher levels of CS-Act than the other sources evaluated.

A summary of a recent pig kidney preparation normalized to 1 kg of ground tissue is presented in Table 2, while the elution profiles of typical column runs are presented in Fig. 1. An overall purification of 100- to 200-fold was obtained based on specific activity. Total units recovered were two- to three-fold greater than had been measured in the starting extract.

Information on the attempted purification of the Con A Sepharose-bound component is also provided in Table 2. The DEAE-cellulose eluate in this case gave two poorly resolved peaks of activity which were pooled separately for further purification. The overall yield of the Con A-bound activity was less than 1% that of the unbound and the final specific activities were only 10–15% of that of the major component. The Con A-bound fractions were therefore presumed to still be impure and have not been analyzed further at this time.

The results of acrylamide gel electrophoreses of the final CS-Act preparation are presented in Fig. 2. Under nondenaturing conditions only a single, somewhat diffuse, protein band running between the insulin standard and the dye front was evident on a heavily loaded gel (Fig. 2a). The accompanying graph depicts the

TABLE 2
Typical Purification of CS-Act from 1 kg of Pig Kidney

	Volume	Protein	CS-Act	Sp act	% Recovery	Purification

Porcine	
dney	Liver
7.6	0.44
3.8	1.6

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TABLE 2
Typical Purification of CS-Act from 1 kg of Pig Kidney

Step	Volume (ml)	Protein (mg)	CS-Act units	Sp act units/mg	% Recovery of units	Purification factor
Heat-treated water extract	2,100	3,900	80,000	19.9	100	1
Con A nonbound octyl-Sepharose eluate	160	270	135,000	504	168	25
DEAE-Sephadex	7	102	257,000	2,500	320	125
S-300	15	82	210,000	2,600	261	130
Con-A bound methylmannoside eluate	140	74	114	1.6	0.14	1[a]
Octyl-Sepharose eluate	21	19	531	28	465[a]	17.5[a]
DEAE-Sephadex						
FX1 (low salt)	1	0.98	128	136	363[a]	85[a]
FX2 (high salt)	1	1.79	286	160		100[a]
S0300						
FX1	0.7	0.61	178	290	416[a]	180[a]
FX2	0.9	0.84	296	352		220[a]

[a] Relative to that in the methylmannoside eluate fraction.

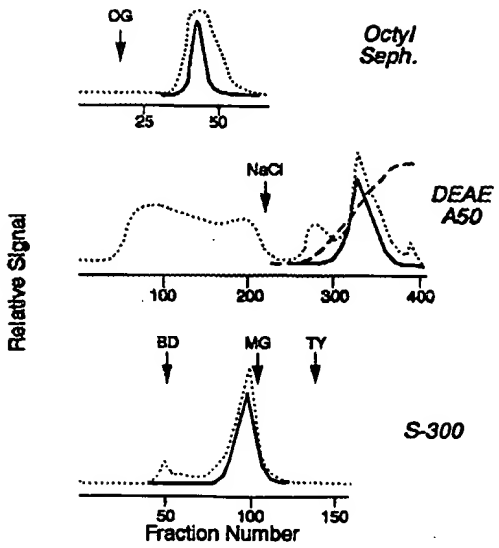
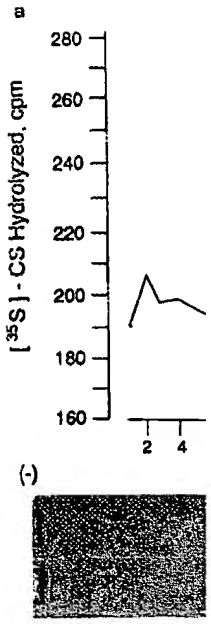


FIG. 1. Chromatographic profiles of the eluates from octyl Sepharose, DEAE cellulose A50, and Sephacryl S-300 columns utilized in the purification of pig kidney CS-Act. Signal strengths have been normalized, with the solid line indicating the CS-Act activity and the dotted line indicating protein estimated from the 280-nm absorption. The arrowheads above the top two profiles indicate the point where the eluant (octyl glucoside, OG, or the sodium chloride gradient, NaCl) was initiated. The broken solid line indicates the measured conductivity in eluate fractions. The arrowheads above the bottom profile mark the elution positions for Blue Dextran (BD), myoglobin (MG), and tyrosine (TY) on a standardization run of the same column.

relative CS-Act activity eluted from slices of an adjacent lane of the same gel. Only slices corresponding to the stained band showed activity. The results of a reduced–denatured sample run on a SDS gel are presented in Fig. 2b. Again the gel was heavily loaded to emphasize impurities. Under these conditions (10% acrylamide) CS-Act migrates in a SDS micelle zone just behind the dye front and can easily be washed from the gel unless it is immediately fixed with a protein crosslinking agent such as glutaraldehyde. Only a small amount of slower migrating material can be seen despite the fact that 40 times as much CS-Act was applied as the standard proteins. At higher gel strengths the CS-Act tends to smear from the SDS micelle zone back into the running gel and does not form a reasonably compact band until gels higher than 15% in acrylamide are employed.

A C_{18} reverse phase HPLC analysis of the same material is shown in Fig. 3. The eluate was monitored for 215 nm absorbing material. Protein and CS-Act activity were also evaluated in fractions collected from the run. CS-Act adhered strongly to the column packing, eluting near the end of the acetonitrile gradient. A number of nonprotein 215-nm absorbing peaks appeared early in the chromatogram. A small inactive protein peak (~5% of the total) was detected at ~20 min and two or three very small active peaks preceded the main component at 29 min. This suggested some minor impurities and a small degree of microheterogeneity in the purified protein. Activator recovery from the HPLC column



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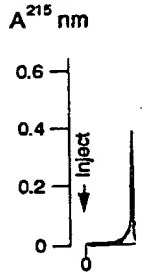

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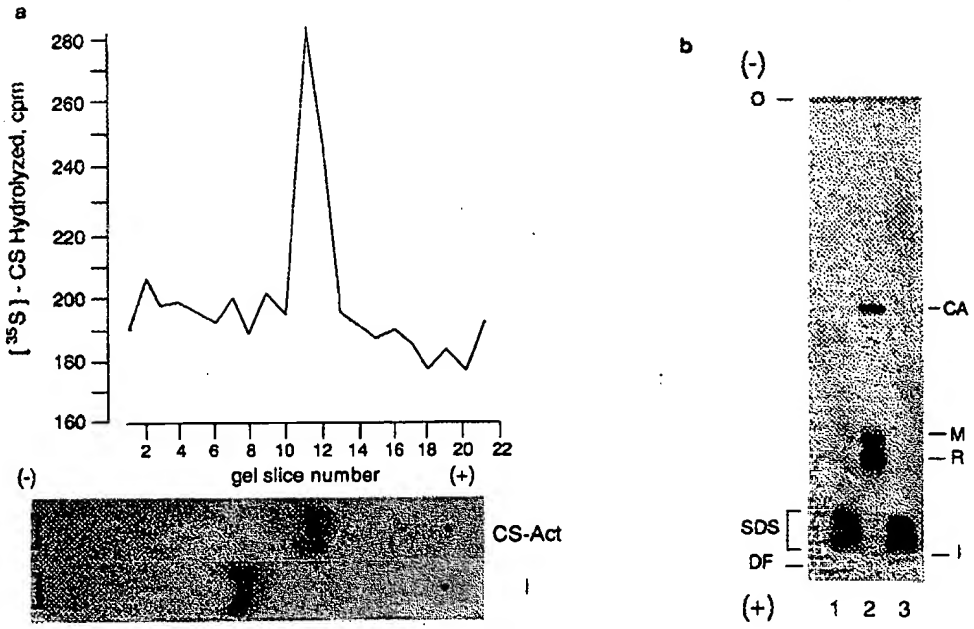

FIG. 2. Polyacrylamide gel electrophoresis of pig kidney CS-Act. (a) CS-Act activity (cpm [^{35}S]sulfate released) of fractions eluted from slices of a polyacrylamide gel electrophoretogram of pig kidney CS-Act. The Coomassie blue staining of an adjacent lane is shown (below) along with that of an insulin standard (I). The electrophoresis was run under nondenaturing (native) conditions as described in the text. (b) SDS gel of reduced and denatured samples, stained with silver after glutaraldehyde fixation. Lane 1 contained 20 μg and lane 3 contained 2.5 μg of pig kidney CS-Act. Lane 2 contained 0.5 μg each of carbonic anhydrase (CA), myoglobin (M), ribonuclease (R), and insulin (I). The positions of the origin (O), dye front (DF), and the SDS micelle zone (SDS) are indicated.

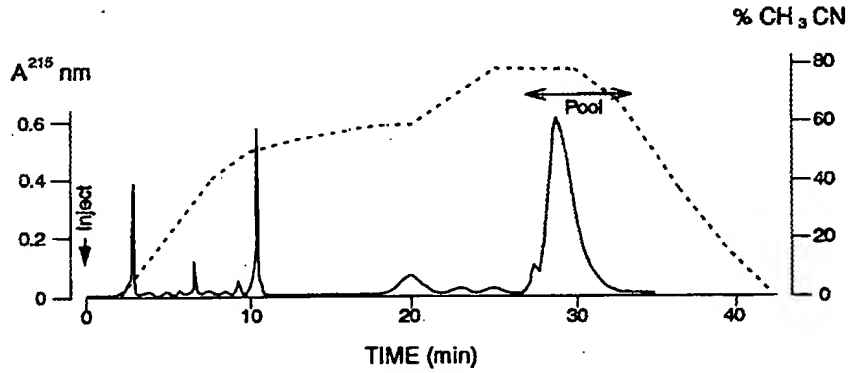

FIG. 3. Reverse phase HPLC analysis of pig kidney CS-Act. The sample in 0.1% TFA was injected onto a 4 × 250 mm C_{18} column and eluted with a programmed gradient of acetonitrile as described in the text. The effluent was monitored at 215 nm. The dashed line indicates the applied acetonitrile gradient. The gradient eluting from the column lagged by 5–8 min.

TABLE 3
Amino Acid Analyses of Pig Kidney CS-Activator and Comparison to Values for the Human Protein

Amino acid	Prep. 1	Prep. 2	Prep. 3	Average	Human[a]	Human cDNA[b]
			mol/10,000 g protein			
Glu/Gln	14.1	12.5	12.4	13.0	13.5	14.1
Asp/Asn	11.6	10.2	11.3	11.0	9.3	8.7
Val	6.5	7.2	7.1	6.9	7.3	8.7
Gly	6.7	5.9	6.2	6.3	5.6	4.3
Ala	5.6	5.9	6.0	5.8	5.5	5.4
Ile	5.2	5.4	5.4	5.3	6.3	7.6
Met	5.8	6.7	2.9	5.1	4.9	6.5
Leu	4.7	5.3	5.1	5.0	4.6	3.3
Ser	4.3	4.1	4.3	4.2	4.9	3.3
Thr	3.7	3.6	4.1	3.8	4.8	4.3
Lys	2.8	3.5	3.3	3.2	3.7	4.3
Arg	2.7	2.7	2.8	2.7	2.4	2.2
Pro	2.5	2.7	2.7	2.6	2.9	2.2
Phe	2.2	2.5	2.4	2.4	2.5	2.2
His	2.2	2.0	2.7	2.3	1.9	2.2
Tyr	1.4	1.8	3.2	2.1	1.9	2.2
Cys	—	—	1.9	—	—	6.5

[a] Average of human liver and urine CS-activator samples analyzed concurrently with prep. 1.
[b] Predicted from the human cDNA amino acid sequence for the putative SAP-1 subunit. The sequence starts from the analytically established N-terminal glycine and runs through lysine 81.

was 65–70% on a protein basis and the peak specific activity had been lowered by 10–15%. (We have experienced difficulty in completely removing CS-Act from some C_{18} columns, with it carrying over into subsequent runs. We now employ C_4 columns and recommend that CS-Act not be applied to C_{18} packings.)

Amino acid analyses on three separate pig kidney CS-Act preparations are presented in Table 3. Human activator values, presented for comparison, are an average of pure liver and urine CS-Act samples analyzed at the same time as preparation 1. The amino acid composition of the pig kidney protein was generally similar to that of human CS-Act. Glutamic and aspartic acids (and/or amides) were the most prevalent residues, followed by neutral aliphatic amino acids. The hydroxy and basic amino acids were less common, with proline and the aromatic residues being in relatively low abundance. An attempt to measure the cysteine content by reducing the protein with mercaptoethanol and trapping thiols with iodoacetate yielded less than one-third of the expected carboxymethyl cysteine content. This was concordant with an unexpectedly low incorporation of radioactivity from ^{14}C-labeled iodoacetic acid in similar reduction–carboxymethylation experiments. The CS-Act protein is apparently extremely difficult to reduce and denature.

Compared to the human protein, the pig kidney CS-Act appeared to contain more aspartic acid/asparagine and leucine, and possibly less isoleucine and threonine. This agrees with sequence differences found on analysis of the first 64

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Pig Kidney: A/C DVCQ
Human cDNA: DNGDVCQ
Rat cDNA: ANEDVCQ

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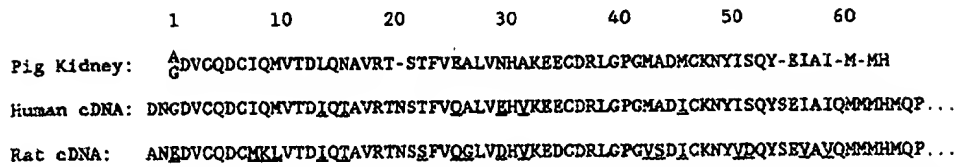

FIG. 4. Comparison of experimentally determined pig kidney CS-Act amino acid sequence with those predicted from the cDNA sequences of the human and rat analogs. Dashes indicate those residues where no standard amino acid could be assigned. Numbers refer to the amino acids numbered from the amino terminus of the experimentally determined sequence. Residues that are different in the human and rat sequences are underlined.

residues (~80% of the expected 81 residues). There were two less isoleucines, one less threonine, and one less glutamic acid/glutamine with two more aspartic acid/asparagine residues and one more each of methionine and leucine.

The experimentally derived amino acid sequence of pig kidney CS-Act over the 66 N-terminal residues is shown in Fig. 4 where it is compared with the sequences deduced from cDNA for human (5) and rat (28) proteins over the same region. The N-terminal amino acid proved to be ambiguous with alanine, glycine, or both being reported on different analyses. This suggests that there may be polymorphism among the population of animals employed in different preparations. The human CS-Act proteins which we have analyzed have had only glycine at this position.

The porcine protein differed from the human at six additional positions within the segment sequenced. These were leucine rather than isoleucine at residue 14, asparagine rather than threonine at position 16, glutamic acid rather than glutamine at position 26, asparagine rather than glutamic acid at position 30, alanine rather than valine at position 32, and methionine rather than isoleucine at position 46. These are conservative substitutions, except for the amide–acid switch at positions 26 and 30 which results in a conservation of functional groups displaced by about one turn of the helix. These alterations are shown on the proposed helical wheel model (6) in Fig. 5.

Pig kidney CS-Act samples contained small and variable amounts of carbohydrate with glucose, glucosamine, mannose, fucose, and sialic acid being detected. In the best preparations glucose and glucosamine ranged between 1 and 2 mol, fucose between 0.5 and 1 mol, mannose between 0 and 1 mol, and sialic acid between 0.2 and 0.3 mol per mol of activator subunit. Occasionally a higher glucose level and 0.5 to 2 mol galactose and galactosamine were also detected. The nature and variability of the carbohydrates in these preparations suggested polysaccharide contaminants or possibly glycolipid ligands accompanying the purified protein.

When the purified activator samples were first subjected to C_{18} reverse phase HPLC, the only sugars detected in CS-Act peak fractions in substantial amounts were glucosamine and fucose. They occurred at approximately 2 and 1 residues per subunit, respectively. Traces of mannose may have also been present but would have constituted less than 0.3 mol per subunit. Carbohydrate values for one of the pig kidney CS-Act preparations is given in Table 4.

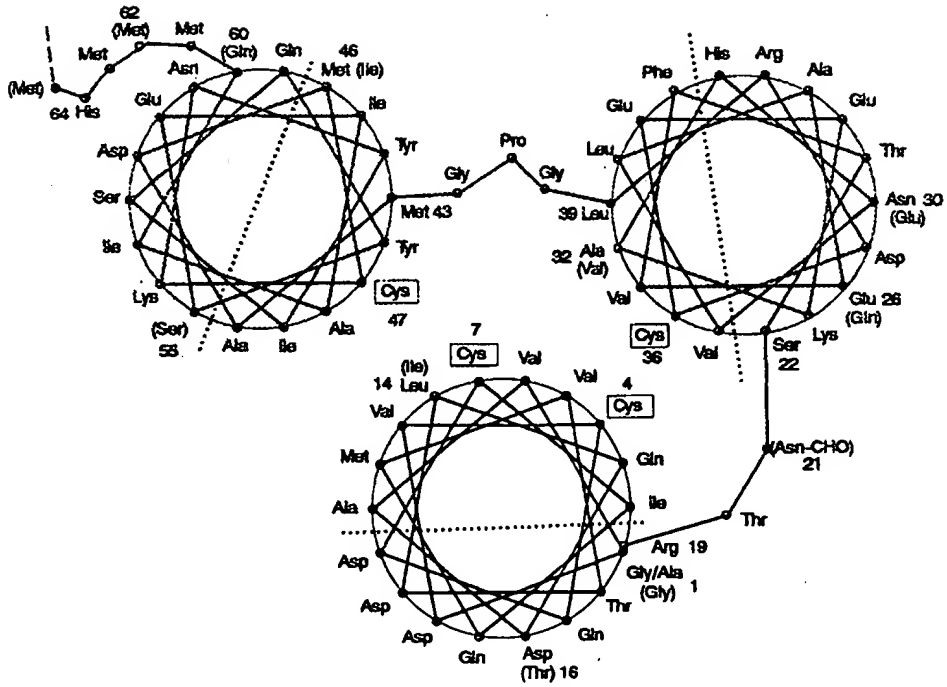

FIG. 5. Helical wheel diagram of the proposed structure for the first 64 amino acids of the pig kidney CS-Act. The first (lower) and third (upper left) helicies project away while the second (upper right) projects toward the observer. Lines through the helicies delineate the predominantly hydrophobic and hydrophilic faces. Parenthetical amino acids are those present in human CS-Act at the equivalent position. Residue numbers are indicated for those amino acids which begin or end a helical segment or are otherwise of note.

Treatment of pig kidney CS-Act under conventional conditions with trypsin, chymotrypsin, or collagenase did not decrease the biological activity. Pronase digestion at a 1:1 ratio of protease to activator protein resulted in a slow decrease in CS-Act activity to about 20% of the initial value over a period of 5 h. After 24 h inactivation was complete (Fig. 6). A parallel decrease in protein content of the sample to about half the initial value was observed.

The apparent molecular mass estimated by HPLC gel fractionation on a TSK

TABLE 4
Carbohydrate Analyses of Pig Kidney CS-Act Preparation 5

	Sugars detected				
Sample	glu	gluN	fuc	man	sial
	mal/10,000 g protein				
PKA-5	2.5	1.3	0.7	1.0	0.3
HPLC-PKA-5	0	2.3	1.2	<0.3	0

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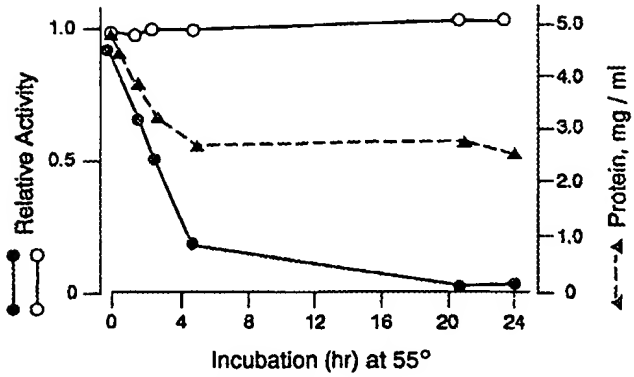

FIG. 6. Pronase digestion of pig kidney CS-Act. Equal quantities of CS-Act and pronase were incubated in 50 mM Tris–Cl, pH 7.5, at 55°C and aliquots analyzed periodically for CS-Act activity and protein. Solid circles indicate relative CS-Act activity and solid triangles indicate total protein. The open circles indicate the CS-Act activity in the control lacking pronase. An apparent half-life of about 2.5 h was observed.

G-2000 SW column varied with the conditions of the chromatography. When the CS-Act storage buffer, 50 mM Tris–Cl, pH 7.5, was employed the apparent molecular mass ranged from 30,000 to 100,000 Da. This lack of reproducibility and large apparent size contrasts with the highly reproducible 20,000–25,000 Da observed on Sephacryl S-300 chromatography during purification (see Fig. 1). The apparent TSK G-2000 molecular mass decreased somewhat with increasing salt concentrations up to 0.5 M NaCl but remained two- to threefold higher than seen on Sephacryl S-300.

When chromatographed in the presence of 0.1% TFA the apparent molecular mass of CS-Act was reduced to 14,000–18,000 Da and activity could be recovered intact from the peak tubes. When analyses were carried out in 50 mM sodium acetate buffer, pH 4.5, the conditions for optimal CS-Act activity, the apparent size was 13,000–16,000 Da. In the presence of 6 M guanidine–HCl, in either pH 7.5 or pH 4.5 buffers, the apparent molecular mass was reduced to 7000–8000 Da, roughly one-half that observed in the absence of the denaturant. After pooling, dialysis and concentration of the 6 M guanidine–HCl peak fractions active activator was recovered which showed the original higher molecular weight on rechromatography at pH 7.5. Examples of typical HPLC gel filtration molecular mass analyses are presented in Fig. 7.

DISCUSSION

While our interest in the CS-Act has focused on its role in the hydrolysis of CS by ARSA, a broader significance for this molecule and its precursor has become evident. First it was established that the CS-Act protein was identical to proteins being investigated as activators for the hydrolysis of ganglioside G_{M1} and globotriaosylceramide by their respective hydrolase systems (2). This implied a broader specificity for binding and solubilizing sphingolipids than had been originally envisioned, and led to the introduction of sphingolipid activator protein 1 or SAP

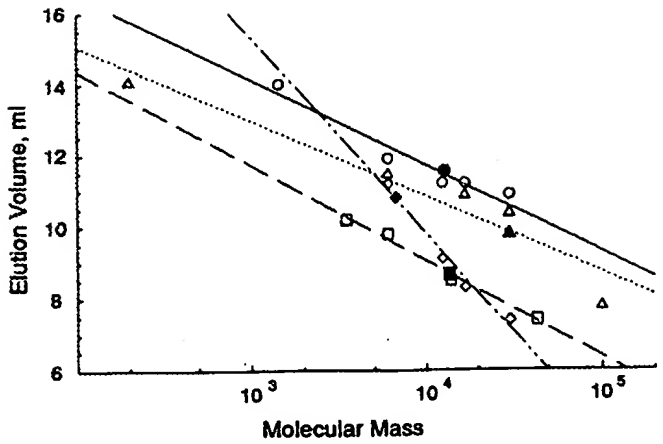

FIG. 7. Molecular mass of pig kidney CS-Act determined by HPLC gel filtration. The open symbols indicate the elution times for standard proteins under various chromatographic conditions. Triangles were in 50 mM Tris–Cl, pH 7.5, 0.15 M NaCl; circles were in 50 mM Na acetate, pH 4.5; squares were in 0.1% TFA; and diamonds were in 6 M guanidine–HCl. The solid symbols indicate the elution times for pig kidney CS-Act. A 7.5 × 300 mm TSK G-2000 SW column was employed and effluents were monitored by either 280- or 215-nm absorption depending on the spectral characteristics of the solvent system.

1 as the name for this protein (3). The activator specificity was further broadened when it was realized that CS-Act could function as well in the enzymatic breakdown of sulfated glycerol glycolipids, the sphingolipid backbone actually not being required (29).

Cloning of the cDNA for the precursor of CS-Act led to the discovery that this protein represented only one of four similar subregions in the original molecule, and that each of these could be processed to a separate sphingolipid activator protein with its own specificity (5). This precursor, now referred to as prosaposin, appears to be a member of a family of proteins which encode multiple subdomains having structures similar to that of CS-Act. This family includes the major glycoprotein secreted by rat Sertoli cells (28) and the precursor to one class of lung surfactant proteins (30). Thus, the studies on the structure and mechanism of action of CS-Act have taken on a much greater significance than originally anticipated.

Almost all studies of CS-Act to date have utilized material isolated from human liver, a source of limited availability. The present purification of the protein from pig kidney in high yield and purity should facilitate the detailed characterization of this fascinating group of proteins.

The present purification takes advantage of two unusual properties of CS-Act to quickly resolve it from most other proteins in the extract. One is heat stability; CS-Act retains its activity and apparently its native structure at temperatures near boiling. The other is a high affinity for matrices with long chain alkyl substituents such as octyl Sepharose. While most membrane proteins bind to these hydrophobic resins, such an affinity is unusual for a small water soluble protein. A negative

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affinity Con A-Sepharose filtration before the octyl-Sepharose column was used in most preparations to remove glycoproteins from the heat-treated extract, although little actual purification was afforded by this step. Conventional ion exchange and gel filtration chromatographies were then employed to remove the remaining contaminants.

The small amount of Con A-binding CS-Act was also purified to some extent through a parallel protocol. A portion of the Con A-bound material eluted from DEAE-cellulose at a higher salt concentration than the major CS-Act fraction. However, neither the low-salt- nor the high-salt-eluting Con A-binding subfractions could be obtained at a purity approaching that of the unbound activator and more detailed characterization of these subforms was not attempted at this time.

The sequence of steps in the purification protocol was designed to quickly free CS-Act from the bulk of the kidney proteins so that activator activity could be assayed accurately. An attempt was also made to minimize the number of dialyses and concentrations needed between columns. After homogenization, heat treatment, and centrifugation, the supernatant was pumped through Con A Sepharose with the effluent flowing directly onto octyl Sepharose. On elution with octyl glucoside only small aliquots of the eluate fractions needed to be dialyzed for CS-Act analysis. The bulk of the active fraction was directly applied to the anion exchange resin where the CS-Act was bound and the uncharged detergent was eliminated with the buffer wash. Desalting of the DEAE-cellulose fractions before Sephacryl S-300 chromatography was not necessary, although concentration was usually needed. The final product was also concentrated before storage.

In developing this protocol, it was noted that the greatest losses of CS-Act occurred on steps involving dialysis and concentration. Yields have been substantially improved by keeping such procedures to a minimum. The eventual introduction of low molecular weight cutoff membranes for both dialysis and concentration has largely eliminated this type of loss. We also found that using resins which had been preconditioned by a cycle of protein absorption and elution minimized activity losses on chromatographic steps, particularly those involving anion exchangers.

The final CS-Act fraction from pig kidney had been purified between 100- and 200-fold from the heat-treated extracts and yielded nearly 100 mg of product per kilogram of tissue. This was 3- to 10-fold more material than we usually obtained from the same amount of human liver, and the starting material was much more readily available. The final specific activity of 2600 U/mg protein was similar to that of our better human CS-Act preparations. This protein constituted about 0.1% of the total pig kidney proteins. Kidneys of several other species examined had 5- to 10-fold lower levels of CS-Act.

The final product seemed to be reasonably pure by several criteria. Rechromatography on Sephacryl S-300 gave a single well-shaped peak with constant specific activity across the peak. On reverse phase HPLC nearly 95% of the protein was in the active peaks. PAGE results were also consistent with a purity greater than 90%, but their interpretation was complicated by the atypical fixation and staining properties of CS-Act and by a propensity for part or all of the protein

to migrate with the SDS micelle band. Perhaps the most compelling indicator of purity was the high quality amino acid sequence which was exceptionally free of spurious peaks. The purity was such that more than 60 amino acids could be sequenced in a single run.

There was evidence for some microheterogeneity. When the preparation was analyzed by reverse phase HPLC, several small peaks preceding the main fraction showed CS-activator activity. Two closely spaced bands with p*I*'s near pH 4 were observed on isoelectric focusing. It is likely that the microheterogeneity resulted from variations in the residual glycosyl substituent at asparagine 21, but differences in the extent of C-terminal processing are also possible.

There was also an indication of some nonprotein contaminants, possibly glycolipids or oligosaccharides, which were removed by reverse phase HPLC. Unfortunately the HPLC protocols have not yet been useful for preparative purposes because of losses in specific activity.

The total amino acid composition and sequence analyses of pig kidney CS-Act were similar to those obtained for human proteins. Over the 60 or so N-terminal amino acids which were identified, ~10% differed between the pig kidney and human proteins. These substitutions were conservative in nearly every case. The two changes which were not strictly conservative, resulted in an interesting acid/amide interchange between residues 26 and 30. These residues are separated by about one turn of the helix proposed for this portion of the molecule. The change preserves the charge and hydrogen bonding possibilities, although it could exert subtle effects on structure or stability. It might, for example, explain a slightly earlier elution of the pig protein on reverse phase HPLC.

We have so far not been able to isolate partial cleavage products from the C-terminal portion of the molecule, and the structure of this part of the pig kidney protein remains unresolved. For comparative purposes it was assumed to be the same as that predicted from the human cDNA sequence.

Carbohydrate analyses showed that only a residual oligosaccharide core remained on the isolated pig kidney CS-Act. The absence of either aspartic acid or asparagine at residue 21 implied that all subunits still carried some sort of glycosyl substituent at this position. However, there was insufficient sugar for these oligosaccharides to be more than two to three residues long. The typical oligosaccharide would be an *N*-acetylglucosamine dissacharide with fucose attached to one of the residues. This is consistent with a typical N-linked oligosaccharide substituent which had been extensively degraded by lysosomal glycosidases. The low sugar content explains the molecules' failure to bind to convanavalin A or to stain with periodate–Schiffs reagent.

Glucose, mannose, and sialic acid were also detected in the CS-Act preparation, but these sugars were removed on reverse phase HPLC. Such HPLC removable sugars could reflect glycolipid ligands which have remained bound to the purified protein, a possibility that should be considered in future studies of this molecule.

While the unusual stability of CS-Act was advantageous in its purification, this property provided problems in the detailed characterization of the molecule. The protein proved so resistant to selective proteolysis by the usual enzymatic and chemical techniques that its protein nature was questioned on more than one

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occasion. Activity was slowly lost on treatment with high levels of pronase, but intermediate products were not detected. Partial cleavage products are probably much more susceptible to proteolysis and therefore never accumulate. Even treatment with SDS and mercaptoethanol at 100°C did not destroy the activity of the protein and only a small portion of cysteine residues were derivatized with iodoacetic acid after such a treatment. It will be important to devise ways to carry out selective degradation of this molecule so that the C-terminal sequence and disulfide bonding pattern can be evaluated. Furst *et al.* have recently reported success in generating C-terminal fragments from the human protein (31).

The molecular mass and subunit composition of CS-Act proved to be somewhat enigmatic. Most reports on the human protein place the native molecular mass around 20,000 Da. In the present study values between 20,000 and 25,000 Da were repeatedly observed for the pig kidney protein on the preparative Sephacryl S-300 columns. However, when purified material was examined by HPLC gel filtration using a TSK G-2000 SW column, results were highly variable with molecular mass ranging from 30,000 to more than 100,000 Da. We interpret this to be due to a concentration-dependent self-association which does not fully reequilibrate over the short time frame of the HPLC technique. Increasing the salt content of the buffer lowered the apparent molecular mass by this technique but it remained near 30,000 Da at neutral pH. When the same material was examined in a mildly acidic system (0.1% TFA or the pH 4.5 activator assay buffer) masses around 15,000 Da were observed. When gel filtration was carried out in 6 M guanidine–HCl at either neutral or acidic pH, the molecular size was reduced to between 7000 and 8000 Da. When the denaturant was removed the higher molecular mass was restored.

These observations are best integrated by the following explanation. The basic CS-activator monomer has a molecular mass around 7000–8000 Da (9200 is expected from the cDNA-derived sequence of the human protein). This subunit exists as a dimer at the acid pH where the protein functions. The dimer is also the dominant species in dilute neutral solutions, but higher order aggregates form as the CS-Act concentration is increased. The kinetics of this higher order aggregation–disaggregation process are relatively slow leading to differences in apparent molecular size for the native protein with various methods of evaluation or different histories of the solutions.

ACKNOWLEDGMENTS

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